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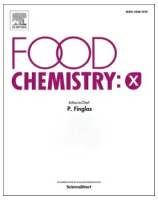

Effects of highland barley β-glucan on gut microbiota composition and metabolism in vitro fermentation

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ABSTRACT

Highland barley β-glucan (HBG) has attracted increasing attention due to its excellent biological activities. However, the effects of HBG on gut flora and metabolites are unknown. Therefore, the effects of HBG on the gut microbiota during fermentation were analyzed by 16 s rRNA sequencing and untargeted metabolomics. The results showed that HBG could enrich microbial diversity, increase the abundance of beneficial bacteria, and inhibit the biology of pathogenic bacteria. In addition, HBG increased the content of short-chain fatty acids and decreased fermentation broth pH. Metabolomics analyses showed that HBG also increased the content of beneficial metabolites such as taurine and affected amino acid metabolism, among other pathways. This study lays the foundation for the application of HBG in functional foods.

1. Introduction

Highland barley (Tibetan hulless barley, *Hordeum vulgare* L.) is a barley cultivar that can thrive in high altitude areas (Guo, Horvath, Chen, Chen, & Zheng, 2020). Referred to as "Qingke" in China, this crop is extensively grown in Tibet, China and serves as the primary staple of the Tibetan community (Lin et al., 2018). The nutritional value of highland barley is noteworthy due to its abundance of bioactive compounds which are characterized by high levels of fiber, vitamins, and protein, coupled with low levels of fat (Guo et al., 2020). In addition, β-glucan is the main active ingredient of highland barley and its content is higher than that of other cereals such as oats (Ren et al., 2018). It has physiological functions such as lowering cholesterol levels, regulating blood sugar levels, antioxidant, anti-inflammatory effects and enhancing immunity (M. Chen et al., 2021). In recent years, the regulatory effects of β-glucan as a soluble dietary fiber on intestinal flora have also received extensive attention and research (Wang et al., 2022).

β-glucan is recognized as a functional substance with potential prebiotic properties (Fehlbaum et al., 2018). It can pass through the human gastrointestinal tract without being broken down by digestive enzymes, and subsequently reach the colon to furnish carbon and energy as substrates for the fermentation of intestinal microflora, thereby modulating the composition of the gut microbiome (Aoe, 2021). β-glucans from different sources have specific glycosidic chains and molecular weights, which affect their physicochemical and biological properties. For example, insoluble β-glucans in yeast and mushrooms usually contain β-(1,3) glycosidic bonds and β-(1,6) branches, while water-soluble β-glucans in highland barley mainly contain β- (1,3) and β- (1,4) glycosidic bonds (Y. Li, You, Liu, & Liu, 2021). Several in vitro and in vivo studies have demonstrated that cereal β-glucan may serve as a microbially fermented substrate that regulates intestinal health by stimulating the production of short-chain fatty acids (SCFAs) and the growth of certain beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* (S. K. Chen et al., 2023). SCFAs (specifically acetic acid, propionic acid, and butyric acid) are the primary metabolites generated through the intestinal microbial fermentation of β-glucan. The SCFAs provide crucial health benefits including safeguarding the intestinal barrier, hindering inflammatory reactions, enhancing insulin resistance, and preventing pathogen proliferation by reducing the pH of the intestinal environment (Ashaolu, Ashaolu, & Adeyeye, 2020).

Currently, studies on the metabolites of intestinal microorganisms in the fermentation of β-glucan is mainly focused on SCFA (N. Liu, Zou, Xie, Meng, & Xu, 2023). However, there are few studies on the changes of other metabolites and metabolic pathways. This research utilized non-target metabolomics with intestinal flora to investigate the in vitro fermentation and metabolic process of β-glucan derived from highland

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barley. This approach enables a comprehensive understanding of the association among highland barley β-glucan, intestinal flora and intestinal flora metabolites. This is advantageous for exploring the mechanism of its in vitro fermentation and provides a theoretical basis for highland barley β-glucan application. It also paves the way for its advancement and utilization.

2. Materials and methods

2.1. Materials

Highland barley were obtained from Chaiqiyuan Special Food Store (Chengbei District, Xining City of Tibet Province, China), trypsin was purchased from Coolaber Technology Co., Ltd. (Beijing, China), thermostable α-amylase (4000 U/g) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2. Preparation of β-glucan from Highland barley

The β-glucan from highland barley (HBG) was performed according to the methods described by Dong with some modifications (Dong, Yang, Zhu, Shen, & Zhang, 2020). Highland barley was ground and sieved through an 80-mesh screen. Anhydrous ethanol (1:10, *w/v*) was mixed with the sieved flour and refluxed at 85 °C for 2 h. Filter and dried overnight after condensation reflux. The dried powder was then soaked in distilled water (1:20, w/v) and the pH was adjusted to 10.0. The mixture was heated at 90 °C for 2 h to facilitate extraction. The resulting solution was centrifuged at 3100 ×*g* for 10 min, and the supernatant was collected. Amylase and trypsin were added to the supernatant to degrade starch and protein. The pH was then adjusted to 4.5, and the solution was refrigerated overnight at 4 °C. After centrifugation, the supernatant was collected. The pH of the supernatant was adjusted to 7.0 and the solution was concentrated under reduced pressure. Ethanol was added to the concentrated solution to achieve an 80 % ethanol concentration and the mixture was left overnight at 4 °C. The precipitate formed was collected by centrifugation and redissolved in distilled water. Ethanol was added to the solution again and its concentration reached 20 %. After standing overnight, the precipitate was centrifuged again. The precipitate was dissolved in distilled water and dialyzed using a membrane with a molecular weight cut-off of 8000–14,000 KDa for 72 h. The β-glucan was obtained by freeze-drying.

2.3. In vitro fermentation by human fecal microbiota

2.3.1. Preparations of fermentation culture and fecal inoculum

The fermentation medium, prepared in a 1-l volume, contained 2.0 g peptone, 2.0 g yeast extract, 0.025 g hemin, 0.5 g L-cysteine, 0.5 g bile salts, 0.1 g NaCl, 0.04 g KH_2PO_4, 0.04 g K_2HPO_4, 0.01 g $CaCl_2{\cdot}2H_2O$, 0.01 g $MgSO_4{\cdot}7H_2O$, 2 g $NaHCO_3$, 4 mL resazurin solution (0.25 g/L), 2 mL tween 80 and 10 μL vitamin k. The medium was sterilized at 121 °C for 20 min using an autoclave prior to use. Fresh human stool samples were collected from three healthy individuals with no history of gastrointestinal diseases, who adhered to a balanced diet and had not taken antibiotics for at least three months. The fecal samples from each donor were pooled, homogenized with sterile phosphate-buffered saline (PBS, 0.1 M, pH 7.2) and inoculated with a 20 % (*w/v*) inoculum in an anaerobic chamber. The fecal inoculum was passed through a four-layer sterile cheesecloth, and the filtrate thus obtained was used for the subsequent fermentation of the fecal homogenate.

In vitro simulation of fecal fermentation in the large intestine referred to the method of Wu et al. (2022). HBG was added to the basal medium as a carbon source for in vitro fermentation of feces (H-HBG group, called HBG). 1 mL of fecal homogenate was added to the anaerobic tube, and 9 mL of medium and 200 mg of HBG were added to each tube in turn. Under the same conditions, no HBG was added as the blank control group, and each group was repeated 3 times (H-Blank group, called Blank). The above tubes were placed in an anaerobic box equipped with an anaerobic gas production bag, and anaerobic fermentation was performed at 37 °C. Samples were collected at 0 h and 24 h of fermentation. The above operations were carried out in a sterile anaerobic bench.

2.3.2. Determination of short-chain fatty acids

The 10 mL fermentation sample was centrifuged (5000 rpm / min) and the supernatant was collected. The 5 mL supernatant was added to 1 mL 25 % (v / v) metaphosphoric acid solution, fully mixed and placed in −10 °C refrigerator overnight. After thawing, the sample was centrifuged again to clarify it. The supernatant was then filtered through a 0.22 μm aqueous membrane and prepared for high-performance liquid chromatography (HPLC) analysis. The contents of SCFAs were determined using an Agilent 1200 high performance liquid chromatography equipped (Agilent Technologies Co. Ltd.).

2.3.3. Determination of pH

The pH values of the fermentation broth at 0 h and 24 h were measured using a pH meter.

2.3.4. Analysis of microbial diversity

The fecal fermentation broth was collected in a sterile centrifuge tube. The DNA extract was analyzed using a 1 % agarose gel, and the concentration and purity of the DNA were assessed using a NanoDrop 2000 UV–Vis spectrophotometer. The V3-V4 variable region of 16S rRNA gene was amplified by PCR. The recovered products of PCR amplification were quantified by fluorescence. Double-end sequencing was performed using Illumina ‘s Miseq PE300 / NovaSeq PE250 sequencer.

Fastp (https://github.com/OpenGene/fastp, version 0.20.0) for raw 16S rRNA gene sequencing Software for quality control, using FLASH (http://www.cbcb.umd.edu/software/flash,version 1.2.7) software. The parts are spliced. Using UPARSE software (http://drive5.com/uparse/, version 7.1), according to 97 % of the. Similarity OTU clustering was performed on the sequence and chimeras were removed. Using RDP classifier (http://rdp.cme.msu.edu/, Version 2.2) The species classification annotation of each sequence was performed, and the comparison threshold was set by comparing with the Silva 16 S rRNA database.

2.4. Statistics and analysis

All figures were generated using Origin 2022 software (OriginLab Corporation, Northampton, MA). Data from repeated measures were statistically analyzed in SPSS version 26, employing two-way analysis of variance (ANOVA) followed by Tukey’s post-hoc tests.

3. Results and discussion

3.1. Effects of HBG fermentation on pH and SCFAs

Changes in pH can reflect changes in the fermentation process and pH is one of the important indicators of the fermentation process (Ma et al., 2022). As shown in Fig. 1, changes in pH were evaluated as a reflection of fermentation. It can be seen that the HBG group showed a significant decrease in pH after 24 h in vitro fermentation compared to the blank group. The presence of β- (1,4) glycosidic bonds in HBG renders it more susceptible to hydrolysis and utilization in the fermentation broth (Li, You, et al., 2021). In vitro simulated fermentation induces intestinal microbial fermentation to produce SCFAs, which in turn causes the pH of the fermentation broth to decrease. The decrease of colon pH value has the ability to inhibit the growth of pathogenic bacteria, which is beneficial to colon health.

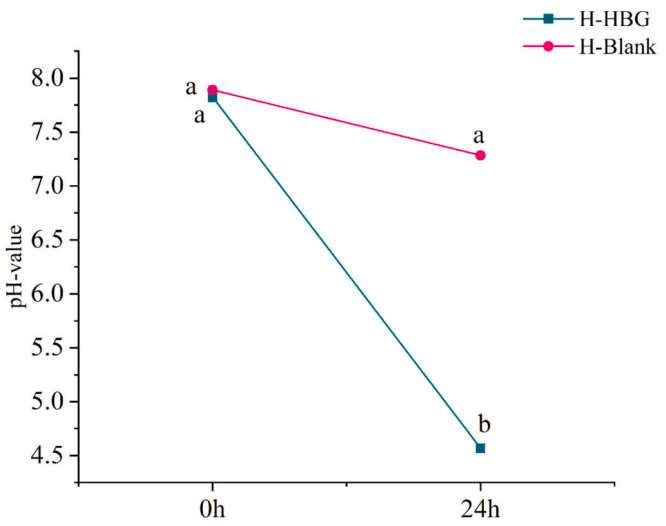

Fig. 1. pH values in fermented cultures with added HBG or Blank. Data are mean ± standard deviation (SD) ($n = 3$). Letters indicate differences ($P < 0.05$) under the same time.

3.2. Effect of the HBG on microbial communities

3.2.1. Effects of HBG on microbial diversity

Chao1 and Observed_species index are indicators that can estimate the richness of microbial communities (Tong et al., 2024). Shannon and Faith_pd index are generally used to evaluate the diversity of communities, Simpson and Pielou_e index are often used to evaluate the uniformity of microbial distribution and Good_coverage is used to evaluate microbial coverage (L. Liu et al., 2022). It can be seen from Fig. S1 that compared with the blank group, the four α-diversity indexes (Chao1, Shannon, Pielou_e and Faith_pd) of the HBG group were significantly reduced. It shows that the richness and diversity of microorganisms have decreased. The decrease of Chao1 index may be due to the enrichment of some bacteria that can utilize polysaccharides in the fecal fermentation broth, which reduces the diversity of microbial communities in the fermentation broth (Zhou, Zhang, Huang, Yang, & Huang, 2020). The β-diversity index was used to analyzed the differences between the samples. The PCoA diagram can intuitively show the similarity and difference among each sample. Fig. S2A is a PCoA analysis based on the Bray-Curtis distance. The abscissa (Axis1,96.4 %) and the ordinate (Axis2,1.9 %) are the two main components that explain the difference among the samples, which can explain 98.3 % of the total variance. It can be seen from Fig. S2A that there was a significant separation between the HBG group and the blank group, indicating that there was a great difference in intestinal microbial composition between the two groups. Similar results were obtained with NMDS analysis and hierarchical clustering trees (Fig. S2B and C). Venn diagram shows 3059 colonies with differences in the HBG group (Fig. S2D). The results of Fig. S2 showed that the microbial community structure of the HBG group was completely different from that of the blank group. The addition of HBG significantly changed the composition of intestinal microorganisms.

3.2.2. Effects of HBG on microbial composition

Gut microbes play a crucial role in health and disease, especially at the phylum and genus levels of their taxonomy. Fig. 2 show the microbiological changes in gate levels in the blank and HBG groups after 24 h of in vitro fermentation. It can be seen that the main microorganisms in the two groups were *Firmicutes*, *Actinobacteria*, *Bacteroidota* and *Proteobacteria*, accounting for more than 90 %. The ruselts showed that compared with the blank group, the abundance of *Firmicutes* in the HBG group was significantly increased and the abundance of *Bacteroidota* was significantly decreased, thereby increasing the ratio of the two (F/B), which is generally considered to be associated with obesity and diabetes and is an important indicator of intestinal flora health (S. Zhang et al., 2021). Among them, *Firmicutes* is considered to produce SCFAs and further promote body health (Xu et al., 2019).These suggest that HBG may reduce the risk of obesity and diabetes by regulating the intestinal flora (X. Li et al., 2020). In addition, the abundance of *Proteobacteria* and *Fusobacteria* also decreased significantly. The presence of these organisms may result in the development of symptoms such as gastric cancer and intestinal flora imbalance. Furthermore, the majority of these organisms are pathogenic to humans, including *Escherichia coli* and *Salmonella* (Li et al., 2021).

It is widely acknowledged that *Lactobacillus* species contribute positively to the intestinal microbiota, enhancing the functionality of the human gastrointestinal tract and aiding in the restoration of intestinal microbiota balance (Li, Wu, et al., 2021). Compared with the blank group, the abundance of *Lactobacillus* in the fermentation broth of the HBG group was significantly increased, and it has become the dominant microorganism in the HBG group (Fig. 2C and D). The results showed that the abundance of *Lactobacillus* increased significantly after fermentation with β-glucan as carbon source, and the enrichment effect of *Lactobacillus* was better than that of other probiotics. In addition, the abundance of *Faecalibacterium*, *Ruminococcus*, *Roseburia* and *Enterococcus* in the HBG group also increased. *Shigella* is a Gram-negative bacterium that can cause human bacillary dysentery (shigellosis), which is characterized by invasion and inflammation of the human colonic epithelium (Pakbin, Bruck, & Bruck, 2023). The results showed that β-glucan fermented in vitro could inhibit the growth of *Escherichia coli*-Shigella and reduce the abundance of *Shigella*. Heatmap analysis showed that HBG had a significant effect on gut microbiota at the genus level. The addition of HBG can significantly increase the abundance of beneficial bacteria and reduce the abundance of harmful bacteria at the genus level. These results indicate that the addition of HBG can change the composition of the flora in the fermentation broth after fermentation and improve the abundance of the flora.

3.2.3. Differential gut microbiota between the blank and HBG groups

Furthermore, LEfSe analysis was utilized to compare shifts in gut microbiota by examining core bacterial phenotypes at the phylum and genus levels between the two groups. As shown in Fig. 3A and B, the blank group was enriched with the *Peptostreptococcus*, *Peptostreptococcaceae*, *Gammaproteobacteria*, *Bacteroides*, *Bacteroidetes*, *Proteobacteria*, *Bacteroidaceae*, *Enterobacteriales*, *Bacteroidia*, *Bacteroidales*, *Tissierellaceae*, *Odoribacteraceae*, *Peptoniphilus* and *Shigella*, while the HBG group was enriched with the *Lachnospiraceae*, *Acidaminococcus*, *Ruminococcaceae*, *Lactobacillus*, *Lactobacillales*, *Faecalibacterium*, *Lactobacillaceae*, *Coriobacterium*, *Bacilli* and *Firmicutes*. The results confirm that many of these trends are highly correlated with the addition of HBG. *Lachnospiraceae* can participate in the process of carbohydrate metabolism thereby producing SCFAs that provide energy to the host (Vandeputte et al., 2017). *Lactobacilli* were more abundant in the *Firmicutes* phylum after HBG treatment, which may be related to the beneficial effects of *Lactobacilli* that can produce SCFAs, lactate, and protect the intestinal barrier (Widyastuti, Febrisiantosa, & Tidona, 2021). Differences and correlations between microbiota of the two groups were analyzed by species taxonomic hierarchical trees and dominant species seed network diagrams (Top 50) (Fig. S3). Compared with the blank group, The main microorganisms enriched by HBG during fermentation are found in the *Firmicutes* included *s_Roseburia_faecis*, *s_Ruminococcus_lactaris*, *s_Clostridium_clostridioforme*, *g_Lachnospira*, *g_Coprococcus*, *g_Blautia*, *g_Ruminococcus*, *s_Ruminococcus_bromii*, *s_Gemmiger_formicilis*, *s_Faecalibacterium_prausnitzii*, *s_Clostridium_celatum*, *s_Lactobacillus_salivarius*, *s_Lactobacillus_ruminis*, *s_Lactobacillus_mucosae* and *g_Enterococcaceae_Enterococcus*. These results indicate that HBG can significantly increase the relative abundance of beneficial bacteria (*Lactobacillus*, *Faecalibacterium*, *Ruminococcus*, *Roseburia* and

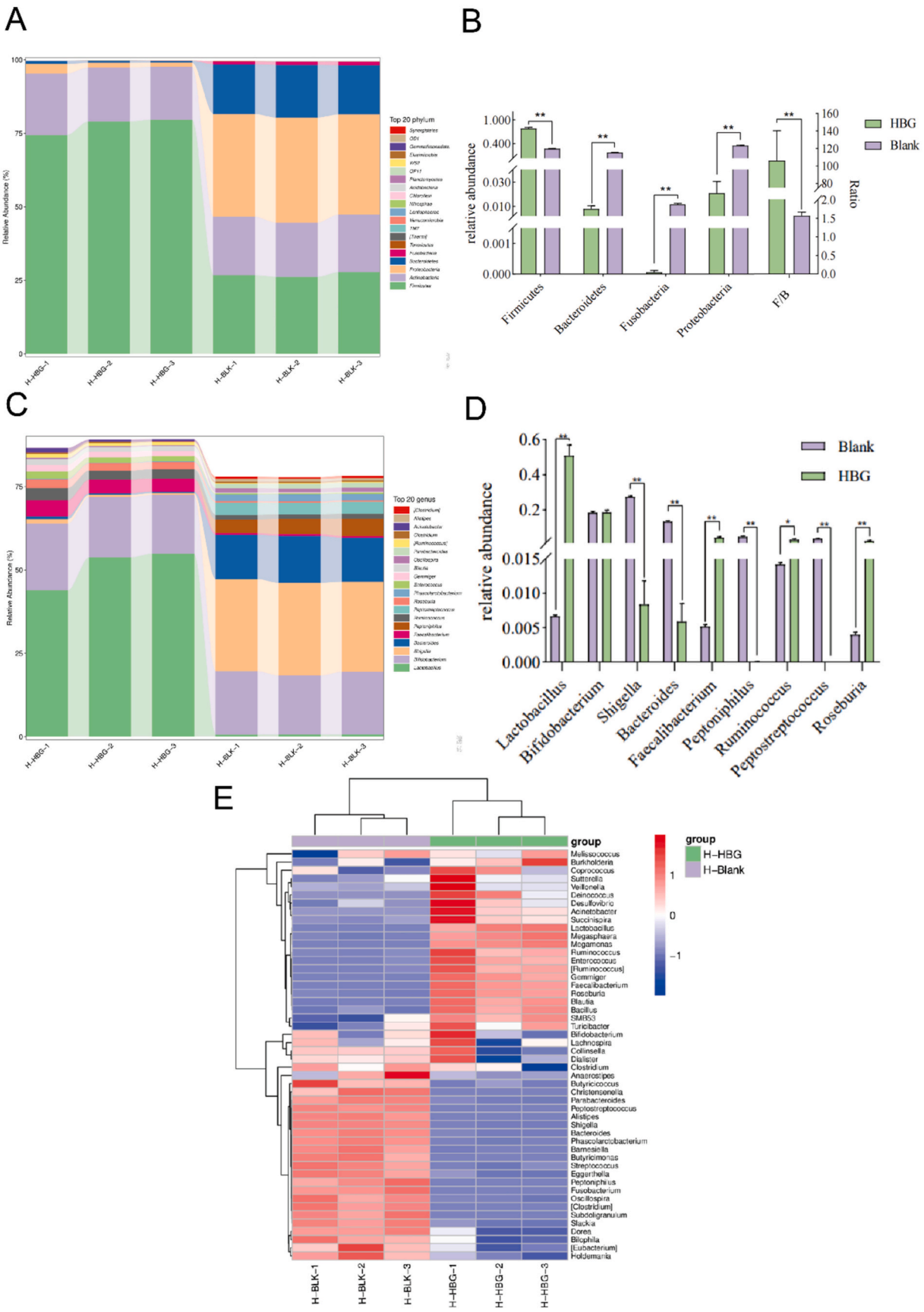

Fig. 2. The relative abundance of fermentation broth at the phylum level (A and B) and the genus level (C and D) after 24 h fecal fermentation, Community heatmap analysis on genus level (E). *$p < 0.05$, ** $p < 0.01$. Data are presented as the mean ± SD (n = 3).

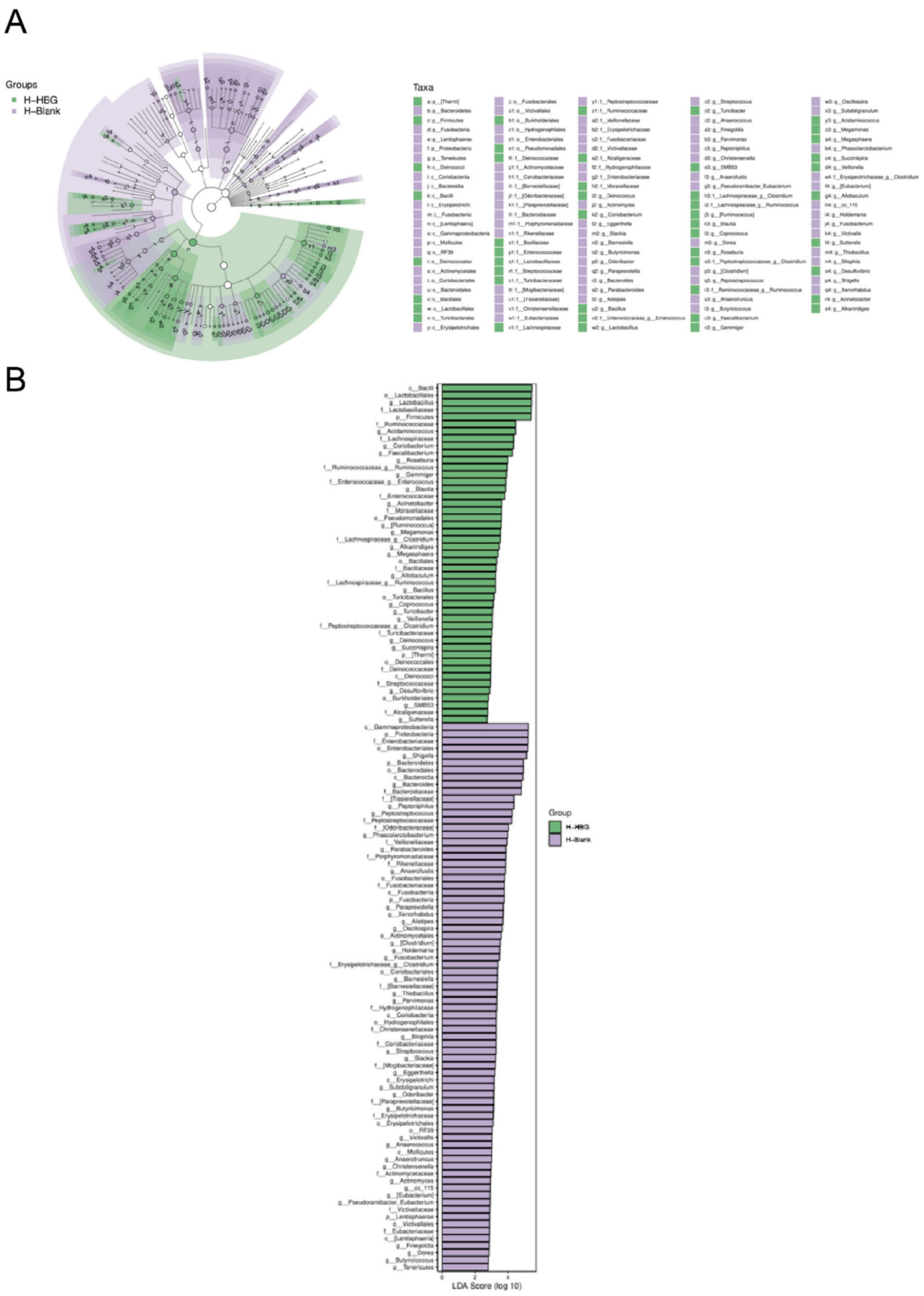

Fig. 3. The LEfSe analysis of HBG and blank groups. Taxonomic cladogram generated by LEfSe analysis (A), LDA scores of taxa enriched at different taxonomy levels (LDA significant threshold = 2) (B).

Enterococcus) and inhibit the relative abundance of harmful bacteria (*Shigella*) in fermentation.

3.3. Effect of the HBG on metabolites of gut microbiota

3.3.1. Screening and identification of differential metabolites

The production of metabolites during in vitro fermentation is closely related to the microbial community (Pakbin et al., 2023). The overall trend of the two sets of samples were obtained by PCA analysis in the positive and negative ion modes. As shown in Fig. S4 A and 4B, there was a significant difference between the HBG group and the blank group. Therefore, differences in metabolites between the two groups could be detected by OPLS-DA analysis. The score plots of the OPLS-DA analysis in positive and negative ion mode are shown in Fig. S4C and S4D. There is a clear difference on the x-axis. The results showed a clear difference in the metabolomic composition of the fermentation samples from the HBG and blank groups. Visualising the differences and contributions of metabolites between the two groups by constructing volcano plots. As shown in Fig. 4A and B, compared with the blank group, 177 metabolites were significantly up-regulated and 119 metabolites were significantly down-regulated in the HBG group. Fig. 5 shows the *Z*-score plots of the relative contents of the two groups of metabolites (Top 30). The relative content of metabolites differed greatly between the two groups and 4-Hydroxycinnamic acid, Indolelactic acid, Baicalein, 4,5-Dihydroorotic acid, Taurine, L-2-Hydroxyglutaric acid, N-Methyl-D-aspartic acid, 11-Dehydrocorticosterone, Methyldopa and L-Malic acid are ranked in the top ten, respectively.

4-Hydroxycinnamic acid is known to have the abilities of regulation blood sugar, anti-inflammatory and antioxidant on human body and indolactic acid can inhibit macrophage pro-inflammatory cytokine (IL-12p70) expression and excessive inflammatory responses by acting on macrophage surface receptors (Beloborodova, Fadeev, & Fedotcheva, 2023). Baicalein is a flavonoid that acts as a specific inhibitor of mammalian liver salivary enzymes, with bacteriostatic, cholesterol-lowering and modulating effects on certain diseases (Y. Y. Li et al., 2022). The above substances play an important role in the anti-inflammatory and antibacterial pathway. Fig. 5 showed that the contents of 4-Hydroxycinnamic acid and Baicalein increased significantly compared with the blank group after in vitro fermentation of HBG. This indicated that the fermentation of HBG can significantly affect the production of 4-Hydroxycinnamic acid and Baicalein in metabolites and thus affect the body 's own anti-inflammatory effects.

Taurine is an amino acid converted from sulfur-containing amino acids, which is mainly distributed in various tissues and organs of the body in free form. Taurine functions as an organic osmolyte, playing a pivotal role in the modulation of cell volume and serving as a precursor in the biosynthesis of bile salts (Baliou et al., 2021). Many studies have found that taurine can participate in lipid metabolism, endocrine activities, anti-inflammatory, hypoglycaemic, regulating lipid digestion and absorption, enhance the body's immune system and other biological functions (Mukherjee, Lordan, Ross, & Cotter, 2020). The results in Fig. 5 showed that the addition of HBG promoted the formation of taurine after fermentation. It shows that taurine can regulate and maintain the normal physiological activities of cells in an acidic environment. N-Methyl-D-aspartic acid is an amino acid derivative that can be used as an excitatory neurotransmitter to participate in the secretory regulation of the hypothalamic-pituitary growth axis (Huang et al., 2021). N-Methyl-D-aspartic acid (NMDA) is an amino acid derivative. The activation of NMDA receptors can promote signal transmission between neurons, thereby enhancing learning and memory. NMDA can stimulate the hypothalamus to release gonadotropin-releasing hormone (GnRH), which in turn affects the hormone secretion of the pituitary gland. NMDA receptors are not only involved in neurotransmission, but may also play a role in neuroprotection. When neurons are damaged, activation of NMDA receptors can induce the expression of protective genes and promote the survival of neurons. After HBG addition and fermentation, the content of *N*-methyl-D-aspartic acid was increased. This indicated that the intake of HBG may affect the regulation of the endocrine system and thus affect the normal physiological activities of the human body. In this study, the contents of Taurine and N-Methyl-D-aspartic acid were significantly increased after HBG fermentation compared with the blank group. The results showed that HBG significantly altered the metabolite composition and increased the contents of beneficial metabolites.

3.3.2. Changes of SCFAs composition during the fermentation

SCFAs are the main metabolites produced by intestinal microorganisms through fermentation, and play an important role in regulating glucose and lipid metabolism and maintaining immune function. Among them, acetic acid, propionic acid and butyric acid were the main SCFAs produced during the fermentation of HBG. The contents of SCFAs during fermentation process are summarized in Table S1. The contents of SCFAs increased from 0.10 ± 0.08 (0 h) to 1.12 ± 0.81 (24 h) mM in HBG group and 0.10 ± 0.06 (0 h) to 0.60 ± 0.85 (24 h) mM in the Blank group. Compared to the blank group, the contents of acetic acid, propionic acid and butyric acid were significantly higher in the HBG group

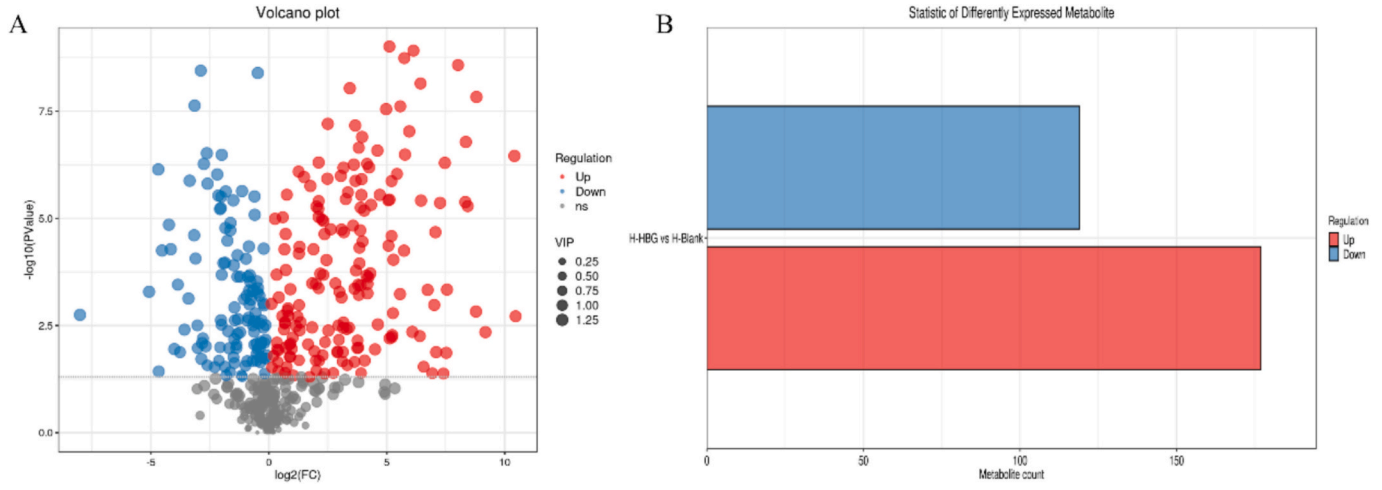

Fig. 4. Volcanic map of differential metabolites (VIP > 1 and $P < 0.05$) (A) (each point: metabolite; red points up-regulated differentially expressed metabolites; blue points represent down-regulated differentially expressed metabolites; grey points represent metabolites that were detected but did not meet the filtering parameters.), Differential metabolite number map (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

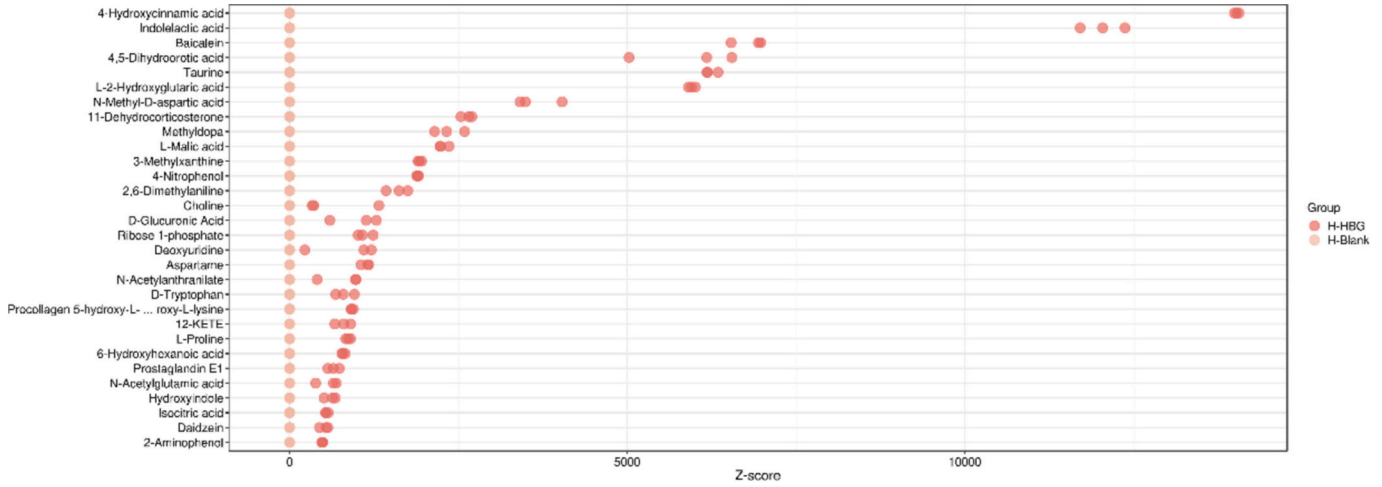

Fig. 5. *Z*-score plot of metabolite contents (Top 30).

after fermentation. Acetic acid was the most abundant SCFA generated by the microbiome (Xiong et al., 2022). Acetic acid was produced in the largest amount compared to other SCFAs. After absorption through the proximal colon, acetic acid is rapidly transferred to the liver to be used as a substrate for cholesterol synthesis and to maintain the normal lipid metabolism process in the body (Rauf et al., 2022). SCFAs, especially butyrate and propionate, can maintain normal physiological activities in the body by modifying tf (transcription factor) and activating specific gpcr (G protein-coupled receptor), thereby regulating cAMP (cyclic adenosine monophosphate) and phosphatidylinositol passage (P. Liu et al., 2021). Propionic acid and butyric acid also bind to g protein-coupled free fatty acid receptors (FFAR)-2 (GPR43) and 3 (GPR41) located at epithelial sites to modulate inflammation and obesit (Deng et al., 2020). Overall, HBG can produce large amounts of SCFAs during fermentation, which is beneficial to human health.

3.3.3. *Metabolite expression in the KEGG pathway*

The representative sequences were annotated in the KEGG database to further analyze the effects of HBG on the function of intestinal microbiome in this pathway after in vitro fermentation. Fig. S5A and S5B showed the main metabolic pathway perturbations induced by the HBG intervention. Top 5 metabolic pathways of importance included by ABC transporters, Biosynthesis of plant secondary metabolites, Central carbon metabolism in cancer, Protein digestion and absorption and Biosynthesis of amino acids. It is worth noting that Protein digestion and absorption and Biosynthesis of amino acids are closely related to gut flora. Proteins are broken down in feces by proteases and various peptidases, which release amino acids that can be used by gut microbes for protein synthesis (Zhao, Zhang, Liu, Brown, & Qiao, 2019). This facilitates recycling between the microbiota and the host. Undigested proteins and amino acids are mainly fermented into various bacterial metabolites such as SCFAs, hydrogen sulphide and ammonia. Some of these bacterial metabolites can be transported within colonic cells and can have beneficial or deleterious effects on these epithelial cells depending on their intracellular toxic potential and concentration. These proteins, amino acids and their metabolites affect changes in the intestinal flora, which in turn affects the regulation of intestinal barrier function and immune defence, and are closely related to host health. These results suggest that the addition of HBG can significantly alter metabolic pathways in organisms.

3.3.4. *Correlation analysis of intestinal flora and metabolites*

In order to gain further insight into the relationship between microbiota and metabolites, correlation analyses were conducted to examine the association between microbiota and differential metabolites in TOP50. As shown in Fig. 6, there are significant differences between different microbial groups and metabolites after HBG in vitro fecal fermentation. It is worth noting that N-Acetylhistidine, Glycitein, Ergothioneine and (+) -cis-lsopulegone play an important role in the metabolites that were significantly correlated and consistent with a wide variety of microbial populations. *N*-acetylhistidine is a kind of histone acid derivatives, it is L-histidine which has an acetyl substituent on the ammonia. *N*-acetylhistidine is synthesized from L-His and acetyl-CoA by histidine acetyltransferase and then hydrolyzed by ammonia enzyme (Atunnise et al., 2024). As a metabolite obtained in the fermentation pathway, *N*-acetylhistidine has been reported to have antioxidant, anti-inflammatory and enzyme activity inhibition effects. In this study, *N*-acetylhistidine was proved to have a significant positive correlation with *Eucterium*, which can produce SCFAs to inhibit pro-inflammatory cytokines and up-regulate anti-inflammatory cytokine levels (X. Zhang et al., 2023). The results showed that the content of *N*-acetylhistidine was closely related to the abundance of Eucterium.

Previous study have reported that HBG could produce a large amount of amino acids and flavonoids during in vitro fermentation, which included Glycitein. (7,4′-dihydroxy-6-methoxyisoflavone)(Diksha & Singh, 2024). Glycitein is a kind of isoflavone compounds. Glycitein has anti-mutagenic and antioxidant properties, which may help protect cells from oxidative stress damage (Diksha & Singh, 2024). In the present study, Glycitein showed a significant positive correlation with *Bacteroides*, which is known as a new generation probiotic.*Bacteroides* play an important role in intestinal immunity and oxidative stress.

Ergothioneine is a trimethyl betaine derivative of histidine (Cheah & Halliwell, 2021a). Ergothioneine can be transported and accumulated by the carrier protein OCTN-1. This property enables ertoamide to effectively target damaged cells and tissues and enhance its therapeutic potential (X. Yang et al., 2023). Ergothioneine can directly react with reactive oxygen species, thereby reducing the damage of oxidative stress to cells, and Ergothioneine can affect the KEAP1-NRF2 pathway to enhance the resistance of cells to oxidative stress (Cheah & Halliwell, 2021b). In the present study, Ergothioneine was closely related to numerous colonies that can produce SCFAs such as *Bacteroides*, *Phascolarctobacterium*, *Oscillospira*, *Butyricimonas*, *Butyricicoccus* have significant correlation. The results suggest that the correlation between Ergothioneine and SCFAs may be critical in regulating redox levels in the organism.

Pulegone, also known as cis-isopulegone, is a monoterpene substance (Chopra & Dhingra, 2023). NLRP3 inflammasome is an important intracellular signaling complex involved in the activation of inflammatory response. By inhibiting ROS signal transduction, pulegone may further inhibit the expression of NLRP3, thereby reducing the

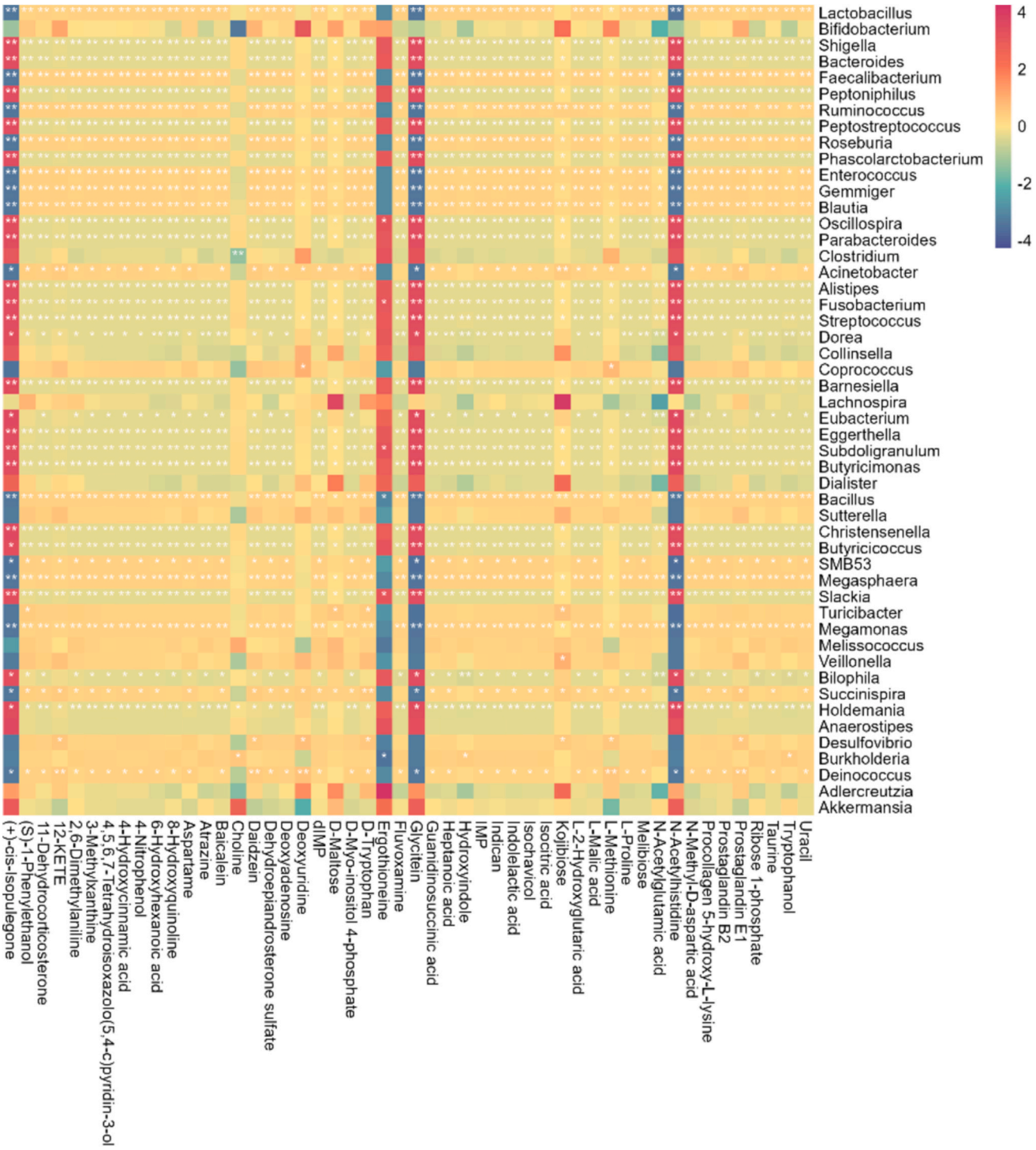

Fig. 6. Correlation between gut microbiota and different metabolites. Red indicates a positive correlation; blue indicates a negative correlation. Significant correlations are indicated by *p < 0.05, **p < 0.01, and marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflammatory response. Pulegone can also show antihypertensive effect by regulating the cholinergic receptor and cyclooxygenase pathway (Q. Yang et al., 2020). This study found that Pulegone had a significant negative correlation with pathogens such as *Enterococcus* and *Bacillus*. This indicates that Pulegone produced by HBG after in vitro fermentation can significantly reduce the abundance of pathogenic bacteria and improve the body ‘s immunity.

4. Conclusion

This study demonstrated that the addition of HBG can enhance the balance of intestinal microorganisms. Specifically, the inclusion of HBG significantly increased the abundance of beneficial bacteria such as *Lactobacillus*, *Faecalibacterium*, and *Ruminococcus*, while inhibiting the growth of pathogenic bacteria, including *Shigella*, *Proteobacteria*, and *Fusobacteria*. Furthermore, HBG fermentation was found to elevate the production of SCFAs. Metabolomic analysis indicated that incorporating

HBG into fermentation processes increased the generation of beneficial metabolites, which subsequently modified the pathways involved in protein digestion, absorption, and amino acid biosynthesis. Additionally, further joint analysis revealed that HBG could significantly enhance the levels of metabolites advantageous to human health, which were closely associated with the production of SCFAs. These evidences suggest that HBG has probiotic properties, which can regulate the composition of intestinal microbiota and enhance the production of beneficial metabolites during in vitro fermentation. It is also proved that there is a certain relationship between gut microbiota and metabolites. In addition, the addition of HBG will affect its own metabolic pathways, thus potentially improving host health.

CRediT authorship contribution statement

Yinchen Ge: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Conceptualization. **Jiaci Liu:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Huacheng Tang:** Resources, Project administration, Funding acquisition, Formal analysis. **Yanqing Zang:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Yang Cao:** Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102089.

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